Heptaplex Real-Time Polimerase Chain Reaction for Neonate Sepsis Pathogens in Brazil

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Abstract

This work has as its main objective the development of a more comprehensive molecular diagnostic method, efficient to diagnose crucial agents in early neonatal bacterial sepsis (ENS), in order to elaborate a rational and specific therapy. Genomic material extracted from the target bacteria *Streptococcus agalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter* sp, *Serratia* sp and *Staphylococcus aureus*, were analyzed by multiplex real-time Polymerase Chain Reaction (qPCR) from samples of DNA from cultures of microorganisms obtained in collection stocks, leukocyte DNA samples from negative and positive control donor individuals. In this study positivity was found by multiplex qPCR in all samples containing the presence of bacterial DNA. The development of this test, based on multiplex qPCR, for the rapid detection (results obtained up to 6 hours) and sensitive of important pathogens causing ENS could potentially outperform in comparison to microbiological methods that are based on bacterial culture. This approach was designed to facilitate progression to specific antimicrobial therapy, which is especially relevant in term and preterm newborns, where empirical treatment is generally used empirically in neonatal intensive care units. The test developed based on Multiplex qPCR proved to be adequate and sensitive for the diagnosis of the presence of genomic DNA of the bacteria studied and that the adaptation of this method has the potential to result in higher rates of positivity to detect the bacteria studied.

Keywords: Neonate sepsis; Multiplex qPCR; Molecular diagnosis; Bacterial pathogens

Introduction

Sepsis is a frequent problem in neonatal intensive care units and can be presented in several clinical ways, involving almost all systems of the body, and being on the list of differential diagnoses or as a possible complication of almost any condition that affects the newborn [1]. One of the most challenging aspects in managing newborns with sepsis is to make their diagnosis. The earliest signs of sepsis are often sudden and non-specific. Most complications of prematurity, including respiratory disease syndrome and congenital heart disease, have similar manifestations to those related to sepsis [2]. The diagnosis of early neonatal sepsis should be as early as possible due to its high morbidity and mortality. Thus, based on this, it is currently based on maternal and neonatal risk factors, clinical manifestations of the newborn and laboratory tests, which lack specificity and sensitivity. Blood culture is the gold standard for the diagnosis of neonatal sepsis [3], but the major problem is that its sensitivity to identify sepsis is only 50% to 80% under ideal conditions [4].The current increase in antibiotic use in the mother has reduced the rate of positivity to levels up to 3% [5]. Thus, a positive blood culture with a pathogenic organism is diagnostic of neonatal sepsis, however, a negative culture does not rule out the possibility of the disease. On the other hand, techniques associated with molecular biology have progressed greatly since the 1950s when DNA had its structure described. Since then, there has been a great deal of interest in the development of molecular technologies for the diagnosis of bacterial sepsis. Most of these tests promise a rapid detection, directly from the blood, with identification before the blood culture and with higher specificity and Sensitivity when compared to the cultures, being able to detect pathogen DNA in very low concentrations [6]. Among the several techniques of molecular biology developed, Polymerase Chain Reaction (PCR) stands out. It is now considered that molecular techniques such as PCR are important methods to detect etiological agents of infection, since they do not require the cultivation of the causal microorganism, being only dependent on the ability to detect its genomic signature. In addition, such techniques have a greater sensitivity when compared to immunological assays and staining methods. PCR can amplify minute amounts of DNA (10 to 100 copies in clinical samples)
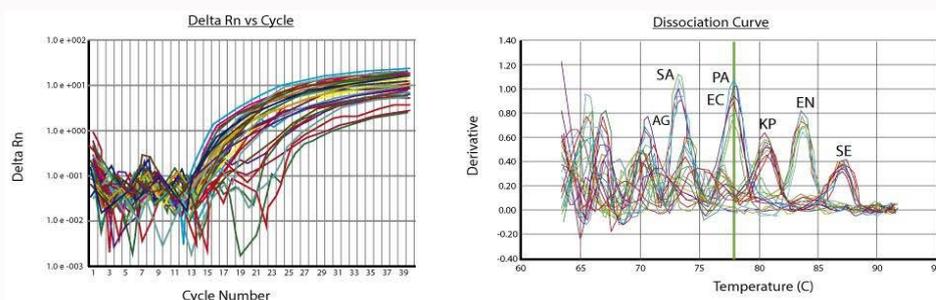
Figure 1: Typical results obtained by qPCR heptaplex. The left graph represents the amplification curves of standard samples and the right graph shows the respective standard dissociation curves from each microorganism obtained from the collection. (SA)=*S. aureus*; (AG)=*S. agalactiae*; (EC)=*E. coli*; (PA)=*P. aeruginosa*; (KP)=*K. pneumonia*; (EN)=*Enterobacter* sp; (SE)=*Serratia* sp.

in a few hours [7].

The major challenge of PCR methods for specific targets is the gap that exists in neonatal infections due to more than one microorganism. As a result, researchers and laboratories have described the Multiplex PCR Diagnostic System, in which the DNAs of several putative bacterial microorganisms in the pathogenesis of neonatal infections can be amplified simultaneously. The use of Multiplex PCR can differentiate several etiological agents responsible for sepsis, based on the differentiation of target amplicons selected by difference in their molecular weight. These have contributing to the simultaneous detection of an increasing number of diseases, from increasing types of clinical samples, with smaller amounts of sample [8-10].

Identification of the microorganism causing the infection and the earliest possible start of the appropriate antibiotic is considered essential. Inadequate initial antibiotic therapy in sepsis is associated with increased mortality by up to five times. On the other hand, indiscriminate antibiotic therapy is responsible for the growing emergence of multi resistant bacteria and fungal infections [11].

There is currently no test or combination of available tests with sufficient specificity or sensitivity for the diagnosis of early neonatal sepsis [12]. In this context, this work has as its main objective the standardization of a new molecular method capable of diagnosing the main agents in early neonatal bacterial sepsis in a developing country, in order to contribute to the elaboration of a more rational and specific therapy.

Materials and Methods

Oligonucleotide primer selection

The oligonucleotide pairs of the primers, as well as their respective product sizes, were designed and described in a previous study published in 2016 by Silva-Junior [10]. The primers were prepared and selected from specific genomic sequences contained in the National Center for Biotechnology Information (GenBank) database to detect the presence of DNA from the seven bacteria (*Streptococcus agalactiae, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter* sp, *Serratia* sp and *Staphylococcus aureus*) [13,14]. All elaborate primers were analyzed by BLASTn software before being synthesized by Integrated DNA Technologies, Brazil.

Genomic DNA extraction

The genomic DNA of the analyzed specimens was extracted using the Illustra Genomic Prep mini spin kit (GE Healthcare, Piscataway, NJ, USA), following the manufacturer's recommendations. The

Figure 2: Typical result of silver stained polyacrylamide gel electrophoresis, from heptaplex PCR, for the diagnosis of seven bacterial specimens obtained from collection. Wells: (M)=50 pb DNA ladder Marker; (1)=*S. agalactiae*; (2)=*E. col*; (3)=*Enterobacter* sp; (4)=*S. aureus*; (5)= *Serratia* sp; (6)=*P. aeruginosa*; (7)=*K. pneumonia*; (8)= heptaplex primer mix.

extracted DNA was quantified in Gene Quant spectrophotometer (GE Healthcare, Piscataway, NJ, USA) at 260 nm.

qPCR multiplex

For real-time multiplex PCR, the SYBR Green PCR core reagent kit (Invitrogen Life Technologies, Carlsbad, CA, USA) was used. The amplification reactions of qPCR followed the protocol described by Anbazhagan et al. [15]. with modifications, being carried out with 3 pmol of each primer, 10 ng of genomic DNA from clinical specimens, 12.5 µL of SYBR Green PCR Master Mix (Invitrogen Life Technologies, Carlsbad, CA, USA), and deionized water to achieve a total final volume of 25 µL of reaction. Reactions were performed under standard thermo cycling conditions of ABI Vii A7 equipment (Applied Biosystems, Warrington, UK). The melting curves were generated to verify the specificity of the amplicons in the positive and negative controls, and consequently analyzed in the equipment software.

Culture of bacterial pathogens

All the isolates of the bacteria studied were selected from the collection cultures obtained in the Microbiology Sector of the Regional Hospital of MatoGrosso do Sul during the year 2014. All the pathogens were cultured in specific media at 37°C for 24 hours before extraction. As a negative control, ultrapure water (Mili-Q) was used autoclaved. As a positive control, total nucleic acid purified from the microorganisms was used, by colonizing each bacterial species in a flask with 1 ml of lysis solution and then extracting the DNA.

Ethics considerations

The study was approved by the Human Research Ethics Committee of the Federal University of MatoGrosso do Sul (UFMS) under protocol n°355.636/CAAE 16465613.7.0000.0021 on 08/08/2013.
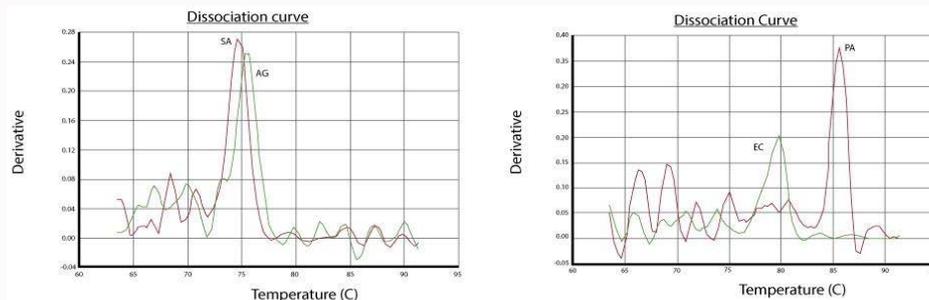
Figure 3: Standard melting curves obtained by the PCRtr after the modification of the primers, demonstrating differentiation between *Staphylococcus aureus* (SA) and *Staphylococcus agalactiae* (AG) and between *Escherichia coli* (EC) and *Pseudomonas aeruginosa* (PA).

Results

Bacterial DNA samples from the standard cultures (positive controls) were tested for the presence of the seven microorganisms and the results of the amplification of the target fragments demonstrating the specificity of the selected primers by amplification and dissociation curves in the multiplex qPCR (heptaplex), may be seen in Figure 1. Furthermore, Figure 2 depicts the polyacrylamide gel performed to demonstrate the primer-specific bands of the seven bacteria selected for diagnosis. In Figure 3 it is possible to observe the dissociation curves obtained before and after the modification of the size of the primers for the diagnosis of microorganisms *S.aureus* and *P. aeruginosa*.

Discussion

The incidence of sepsis in the neonatal period is higher than in any other period of life and varies from hospital to hospital, especially in poor countries. The average incidence of proven neonatal sepsis per culture is 16 per 1,000 live births, according to Satar and Ozlu [16]. According to these same authors, proven neonatal sepsis by culture ranges from 2.2 to 9.8 per 1,000 live births, however the incidence of clinical sepsis varies from 20 to 50 per 1,000 live births. Neither the treatment nor the neuro developmental outcomes of neonates surviving sepsis have changed significantly in the past 30 years, despite the great efforts to reduce the high rates of neonatal infection, which has occurred in the context of major advances in other areas of the neonatal care such as nutrition, respiratory distress management and pulmonary hypertension, and the use of therapeutic hypothermia in hypoxic-ischemic encephalopathy [17]. Thus, it is justified the search and improvement of new diagnostic techniques, among which the molecular ones, which, because they have been shown to be faster, more specific and sensitive in the diagnosis of neonatal sepsis, have the potential in the future to replace the current microbiological method.

In addition, rapid diagnosis combined with early antibiotic therapy and appropriate management of metabolic and respiratory problems can significantly reduce future problems related to ENS [18]. The microbiological culture of the blood is still considered the gold standard in the diagnosis of neonatal sepsis, but its sensitivity is usually low in neonates, not counting its delay from 48 to 72 hours for its result to become positive [19]. In a study conducted by our group the blood culture positivity rate was 3% [12], similar to that found by Al-Taiar, et al. [20], who observed a positivity rate of 4.6%. Other studies reported higher rates of positivity in their blood cultures, such as the 23.59% found by Patel, et al. [21] and the 42% observed by Hussein and Khaled [21,22]. Thus, it can be observed that a negative result in a blood culture does not necessarily imply the absence of bacterial sepsis in a neonate, which justifies the difficulty of isolating the microorganism even when present in the bloodstream. All this makes it increasingly necessary to implement molecular biology techniques, such as PCR, because it is a faster and more specific method for detecting the presence of the microorganism in the blood, making it an excellent tool in the aid and treatment of these patients. Considering the magnitude of the problem and the great impact in terms of morbidity and mortality that the episodes of ENS result for both the patients and the health systems, in this study, genomic DNA determination of common microorganisms was performed in neonatal sepsis through multiplex qPCR, in order to provide support for a more agile and effective conduct in favor of a correct and early diagnosis, as well as in the elaboration of infection control measures for these patients. The use of PCR for the evaluation of children with sepsis admitted to neonatal intensive care units can reduce the rates of empirical antibiotic therapy and length of hospital stay, since qPCR is a method that, besides being capable of diagnosing several bacteria simultaneously, is faster, more accurate and more sensitive than conventional PCR and the microbiological culture [12,23-25].

In this study, multiplex qPCR positivity was found in all samples with bacterial DNA. It should be noted, however, that a positive result for the presence of genomic DNA in the blood does not necessarily mean that the patient is presenting with sepsis by that bacterium, and may suggest only a possible bacterial colonization. However, the risk of true sepsis increases dramatically when such results occur in a patient with risk factors and clinical signs of infection.

There are fragments that even of different sizes still face conditions in which one amplicon overlaps another, as observed in Figure 1. Thus, it was not possible through the first multiplex test to differentiate the bacteria *Pseudomonas aeruginosa* and *Staphycoccus aureus* due to their close proximity to the melting temperatures of these bacteria in the amplification and dissociation curves. This required resizing of the target fragment so that there was a clear separation and the diagnosis was specific. Such differentiation was achieved by reducing the size of the primer antisense fragment for *Staphylococcus aureus* and the first sense for *Pseudomonas aeruginosa*, providing a modification in the melting temperature of the amplicons of these bacteria as shown in Figure 3. Also, according to our experience, it is very important to consider that the reaction volumes in the multiplex qPCR can interfere in the variation of the positioning of the peaks of the dissociation curve, avoiding also working with more concentrated samples or with different buffers, which can generate confusing and overlapping peaks. To establish a methodology with larger volumes and standard curves with microorganisms of collection or archive,

as in this study, were also fundamental factors for the success of the heptaplex reaction.

The development of this new multiplex qPCR-based assay for rapid detection (up to 6 hours) and sensitive detection of important pathogens causing neonatal sepsis may potentially outperform in comparison with microbiological methods based on microbiological culture. All this in order to facilitate progression to specific antimicrobial therapy, which is especially relevant in term and preterm newborns, where empirical treatment is generally used empirically in neonatal intensive care units [26]. The methodology applied here has already been used, demonstrating an effective tool for bacterial diagnosis in a population study carried out in neonates with clinical diagnosis of early sepsis [12]. It can be concluded that the developed test, based on qPCR, has been shown to be adequate and sensitive for the diagnosis of the presence of genomic DNA of the bacteria studied and that the adaptation of this method has the potential to result in higher rates of positivity to detect bacteria researched. Thus, the potential of the molecular test described, combined with the clinical practice of the patients, besides having great potential for epidemiological studies may also justify its use in the early diagnosis of neonatal sepsis.

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